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(54) Title: RESAZURIN-BASED CYTOTOXICITY ASSAY

(57) Abstract: The method of the present invention relies on culturing hepatic cells in a growth medium in the presence of resazurin and a cytotoxic compound for a period time, and then measuring the degree of fluorescence of reduced resazurin as an indicator of nonviable cells. The assays described herein are particularly useful in screening drugs to determine their cytotoxic potential. Most commonly, drugs would be screened by the methods described herein to determine their potential hepatic toxicity as an alternative to current in vitro or in vivo models of liver toxicity. Alternatively the methods described herein could be used to determine the chemotherapeutic efficacy of drugs, particularly those directed towards hepatic tumors.

TITLE OF THE INVENTION

RESAZURIN-BASED CYTOTOXICITY ASSAY

CROSS-REFERENCE TO RELATED APPLICATION

This application claims the benefit of United States Provisional Application Serial No. 09/587,007, filed June 20, 2000.

BACKGROUND OF THE INVENTION

Resazurin is a dye reported to be useful for quantitatively measuring cell-mediated cytotoxicity, cell proliferation and mitochondrial metabolic activity and is sold under the name AlamarBlue™ (a trademark of AccuMed International, Inc.). AlamarBlue™ is a proprietary mixture that is marketed as an indicator dye for cell proliferation and cytotoxicity assays containing resazurin, and other dyes (poising agents) added to inhibit non-specific reduction of resazurin, described in United States Patent 5,501,959. Resazurin is reduced by respiring mitochondria to form resorfurin, and is a more sensitive reporter than other commonly used mitochondrial reductase dyes such as MTT and XTT. MTT and XTT are reduced by components early in the respiratory chain, and ultimately block electron flow and respiration. AlamarBlue substitutes for molecular oxygen as an electron acceptor for oxidoreductases, such as cytochrome oxidase, the last cytochrome in the respiratory chain; accepting electrons at this last step does not interfere with respiration. AlamarBlue™ reduction leads to a loss of oxygen and a gain of hydrogen in the molecule, and its reduced form can be detected both colorimetrically and fluorometrically. Relative to most other reductase indicator dyes, AlamarBlue™ is non-toxic, and insensitive to drugs, serum and phenol red. Resazurin alone may be as useful as the AlamarBlue™ mixture for following proliferation of certain types of cells (Rasmussen, 1999).

Current cytotoxicity assays based on mitochondrial function are fundamentally cell-proliferation assays, and proliferation can be inhibited independently of frank cytotoxicity. For example, differentiation of cell lines (such that the cells are viable, but are no longer proliferating) can be interpreted as toxicity with such assays. A second complication is that mitochondria numbers can increase independently of cell numbers. To avoid confounding results from reductase-based cytotoxicity assays, assays are needed that reflect cell death *per se* rather than change or loss of functions associated with healthy cells.

The current resazurin-based cytotoxicity assay consists of adding a composition of resazurin to an active cell culture under investigation, allowing the cells to proliferate for a short period of time, typically one hour, and then measuring the reduction of resazurin colorimetrically (color change from blue to red) or fluorometrically (resorfurin excites strongly at 580 nanometers). An increase in the signal generated by the resazurin is indicative of a proliferative cellular state. Thus when a non-proliferative cell sample is compared to a proliferative sample, the resazurin signal is lower.

The present invention describes a novel assay method using resazurin as an indicator of cellular toxicity. The present invention describes a method under which long-term exposure of the resazurin dye to a cell population produces a more pronounced index of cytotoxicity. This novel cytotoxicity assay shows increased AlamarBlue™ reduction associated with cell death, as opposed to cell proliferation, and importantly appears independent of viable cell number and proliferative state of the cell population. Viable cells are capable of clearing the initial resorufin formed after an initial resazurin exposure, and this decrease in background fluorescence serves to amplify the signal associated with cytotoxicity.

SUMMARY OF THE INVENTION

The method of the present invention is drawn to a method which comprises culturing cells in a growth medium in the presence of resazurin and a putative cytotoxic test compound for a period time, and then measuring the degree of fluorescence of reduced resazurin as an indicator of nonviable cells.

The assays described herein are particularly useful in screening drugs to determine their cytotoxic potential. Most commonly, drugs would be screened by the methods described herein to determine their potential hepatic toxicity as an alternative to current *in vitro* or *in vivo* models of liver toxicity. Alternatively the methods described herein could be used to determine the chemotherapeutic efficacy of drugs, particularly those directed towards hepatic tumors.

BRIEF DESCRIPTION OF THE DRAWING

Figure 1 - Panels A and B. AlamarBlue™ fluorescence responses from cells treated for 1 day (white bars) or 3 days (black bars) with cisplatin (cis-P), transplatin (trans-P), ethionine (ethio), methionine (methio), 5-fluorouracil (5FU), flufenamic acid (fluf), or diflunisal (diflu) prior to AlamarBlue™ addition. Concentrations are μM. Control data are pooled from wells treated with media or 0.3% DMSO (vehicle for fluf and diflu). A) Exposure to AlamarBlue™ for 1 hour. B) Exposure to AlamarBlue™ for 3 days. Data are pooled from three experiments.

Figure 2 - Time course for changes in AlamarBlue™ fluorescence. Cells were treated with toxic compounds for 3 days prior to AlamarBlue™ addition. AlamarBlue™ exposures were 1 day (gray bars), 2 days (black bars) or 3 days (white bars). Note the decreasing fluorescence over time in control

wells and with low concentrations of drugs. Data are pooled from 2 experiments (8 wells for each condition). Abbreviations are as given in Figure 1.

Figure 3 - Panels A and B. Comparisons of fluorescence changes of AlamarBlue™ (white bars) and Resazurin (black bars) in A) Short-term (1 hour) assay and in B) Long-term (3 day) assay. Treatment with drugs was for 3 days prior to exposures to AlamarBlue™ (1:100 final dilution) or Resazurin (5 uM). Data are pooled from 2 experiments. Abbreviations and units are as given in Fig. 1.

Figure 4 - Panels A and B. Antioxidant Treatment does not prevent reduction (increase in fluorescence) of: A) Dihydro-fluorescein Diacetate (DHFDA) or B) AlamarBlue™. Cells were exposed to 5-fluorouracil (5FU), its control 5-chlorouracil (5ClU), flufenamic acid (fluf) or diflunisal (diflu) for 1 day prior to antioxidant treatment (white bars, n-acetylcysteine 3 mM, tocopherol phosphate 100 uM, and ascorbate 100 uM) or vehicle (black bars). Exposure to vehicle, antioxidants, DHFDA and AlamarBlue™ was for 3 days. Data is from one representative experiment.

Figure 5 - Panels A and B. Effects of an electron coupler (phenazine methosulfate, PMS) on AlamarBlue™ reduction (fluorescence increase) at: A) 1 hour and B) 3 days. HepG2 cells were exposed for 3 days to cisplatin (cis-P) and transplatin (trans-P) at indicated concentrations (uM), and PMS and AlamarBlue™ were added. Data is pooled from 2 experiments.

Figure 6 - Panels A and B. Effect of exposure to AlamarBlue™ on subsequent responses to AlamarBlue™. HepG2 cells were exposed to drugs for 7

days with (white bars) or without (black bars) AlamarBlue™ the final 4 days of drug exposure. Media with and without AlamarBlue was then replaced with media plus AlamarBlue and fluorescence was determined at : A) 1 hour and B) 4 days. Concentrations are in micromolar. Data is pooled from 2 experiments.

Figure 7 - Panels A, B and C. Comparison of AlamarBlue™ reduction (increased fluorescence) in growing (50% confluent, white bars) and confluent (black bars) HepG2 cells. Drugs were added at the indicated concentrations (uM) for: A) one day, B) three days and C) seven days prior to addition of AlamarBlue™. Fluorescence was determined after four days exposure to AlamarBlue™. Data was pooled from two experiments at each time point.

Figure 8 - Long-term AlamarBlue™ reduction in primary rat hepatocyte culture. Cells were exposed to drugs for three days prior to addition of AlamarBlue™, and fluorescence readings were taken after two days (gray bars), four days (black bars) and six days (white bars) exposure to AlamarBlue™. Data is from one representative experiment; other drugs also produced increases in AlamarBlue™ fluorescence similar to those obtained in HepG2 cells.

DETAILED DESCRIPTION

The present invention provides, in one embodiment a method to determine the cytotoxicity of a drug comprising:

(a) contacting a culture of hepatic cells with a test drug;

(b) culturing the cells in the presence the test drug;

(c) contacting the cells with a resazurin composition;

(d) culturing the cells in the presence of the resazurin for time sufficient for a first measurable signal associated with the reduction of resazurin to resorfurin by viable cells to weaken, the first signal being selected from the group consisting of fluorescence or colorimetric indication; and,

(e) measuring a second fluorescence as an indicator of nonviable cells.

The hepatic cells useful in the present invention may be primary cell cultures derived from any vertebrate, or may be any stable hepatic cell line. Primary cell cultures, as used herein, refer to cells obtained directly from an animal and are dispersed for assay. Stable hepatic cell lines are those cell lines that can be propagated *in vitro*. Particular hepatic cells useful in the present invention include primary rat hepatocytes, HepG2 (ATCC HB-8065), and primary human hepatocytes. For cells to be useful in this assay, it is important that the cells are capable of clearing the metabolite resorufin from the contacting media. It is readily apparent to those of ordinary skill in the art that a wide variety of cells and cell types are useful in the method of the present invention. Other cells useful for use in the present invention can be determined by practicing the methods of the present invention and observing similar results as those described for liver-derived cells. Other cells and cell types useful in the method of the present invention include but are not limited to cells obtained from cardiac, neural, kidney, muscle, pancreatic, or any other organ of the body of the vertebrate, as well as other tissues of the vertebrate.

Resazurin may be used at a concentration in the range of 0.5-50 μM, preferably at a concentration about 5 μM. The resazurin composition may comprise resazurin as a single compound in a suitable solution. Suitable solutions are those that are not toxic to hepatic or other cell culture such as physiologically buffered saline solutions, or culture media. Physiological buffers are defined as those with a pH in the range of about 6.5 – 7.8. Resazurin compositions containing additional

dyes or poising agents are also suitable for use in the present invention, such as those described in US Patent 5,501,959. The presence of poising agents does not enhance the signal to noise ratio in the methods of the present invention. Moreover the problem of autoreduction, and thus increased fluorescence of resazurin, described in U. S. Patent 5,501,959 is not observed by the methods of the present invention.

On the contrary, resazurin reduced to resorfurin by viable cells is apparently oxidized or otherwise metabolized such that after a period of time any initial fluorescent signal is reduced to near background fluorescence by viable cells in control wells before a second fluorescent signal becomes predominant, which is indicative of nonviable cells in treated wells. The amount of time necessary to culture the cells in the presence of the resazurin sufficient to observe the weakening of the initial fluorescent signal is determined by methods well known in the art, for example by running a time course experiment. A preferred amount of time is at least 20 hours, and more preferably in the range of 20 – 72 hours. Alternatively the first measurable signal may be determined using colorimetric means, and similarly monitored to observe a decrease in the colorimetric indication.

Resorfurin is strongly fluorescing at a wavelength of about 580 nanometers. An excitation source having a wavelength from about 560 nanometers to about 530 nanometers is used excite fluorescence of resorfurin. Although the ex 535/em 580 filter set is known to be optimal for measuring resazurin reduction, we have also observed fluorescence of the accumulating resorufin by exciting with a laser at 488 nm, suggesting utility with non-optimal fluorescent excitation and emission.

The amount of time necessary to culture the cells in the presence of the test drugs or resazurin is determined by methods well known in the art, for example by running a time course experiment. Whether a test compound or a drug being tested is cytotoxic, or the degree of cytotoxicity of a compound or drug is determined by

measuring a second fluorescent signal generated by reduction or other metabolism of resazurin by nonviable cells. Thus by comparing an increase in fluorescence compared to a control culture of cells, a compound or drug may be determined to be toxic.

There are two major advantages of this novel prolonged incubation AlamarBlue™-based cytotoxicity assay. First, the signal is superior to that generally observed in the traditional short-term AlamarBlue™ assay. The pronounced increase in fluorescence associated with toxicity should be easy to detect in high throughput screening of pharmaceutical compounds. Second, in contrast to proliferation assays, ie., XTT, MTT, WST-1 and the traditional AlamarBlue™ assays, the novel assay is not affected by decreased mitochondrial respiration which might reflect decreased proliferation, decreased viability, or even a combination of decreased viability accompanied by an opposing increased stress. Without wishing to be bound by theory, it is likely that the AlamarBlue™ reduction observed after prolonged incubation with toxin-treated cells represents scavenging of electrons from lipid peroxidation cascades in dying cells. Other advantages of this novel assay are the same as noted for the traditional short-term assay; AlamarBlue™ is an "add-and-read" substrate that is relatively non-toxic and chemically insensitive to serum, phenol red and most drugs and antioxidants. Optimally, both short and long term AlamarBlue assays should be run on the same cell culture plates to assess cytotoxicity from two different vantages.

The following examples illustrate the present invention without, however, limiting the same thereto.

EXAMPLES

EXAMPLE 1

Materials: AlamarBlue™ was purchased from Biosource International (Carlsbad, CA), resazurin and dihydrofluorescein were from Molecular Probes (Eugene, OR) and Electron Coupler was from Boehringer Mannheim (Indianapolis, IN). Cisplatin, transplatin, 5-fluorouracil, 5-chlorouracil, ethionine, methionine, flufenamic acid, diflunisal, acetylsalicylic acid, and Triton were from Sigma Chemical Co. (St. Louis, MO). Cells- HepG2 human hepatoma cells were plated in 96 well black clear bottom plates (Polyfiltronics, NUNC) at 90% confluence (unless stated otherwise) in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (GIBCO). Quadruplicate wells were treated 24-48 hours later with test compounds in 100 µl final volume. HepG2 cells were kept at 37 °C in a humidified cell culture incubator in 5% CO2/ 95% air.

Confluent primary rat hepatocytes were obtained from In Vitro Technologies (Baltimore, MD) and were delivered plated in collagen-coated Costar 96 well black clear bottom plates. Cell media was changed to serum-free phenol red-free DMEM plus bovine serum albumin and compounds added as described above. AlamarBlue™ Assay- 100 µl of AlamarBlue™ (diluted 1:50 in Hanks Buffer) was added under sterile conditions to treated 96 well plates at indicated times. Immediately after adding AlamarBlue™, fluorescence readings of the 96-well plate were recorded using a Fluorometric Imaging Plate Reader (FLIPR™, Molecular Devices, Sunnyvale, CA). FLIPR uses an argon laser (488, 0.5W) to excite the plate while collecting images from each well with a cooled CCD camera (emission filter band pass 510-570 nm). The emission intensity is expressed as relative fluorescence units (FLU). The initial zero time point readings (which averaged about 6000 FLU and were essentially equal to readings from an empty culture plate) were subtracted from later readings. Readings of 60,000 FLU saturated the CCD camera, but

maximal levels seldom exceeded 45,000 FLU. In three experiments, resazurin (10 uM in Hanks Buffer) was added for comparison with AlamarBlue™.

Fluorometer readings also were taken from pooled control and ethionine-treated samples to determine wavelength ranges for excitation and emission of reduced AlamarBlue™. Setting emission at 600 nm, the fluorescence increased with excitation from about 510 nm to a shoulder at 530 nm to a peak at 572nm. When excited at 530 nm, a pronounced emission peak was observed for the ethionine sample at about 585 nm, in agreement with reported values for reduced AlamarBlue™ and resazurin. A broad shoulder was observed at higher wavelengths for the samples and oxidized AlamarBlue™ in media with a second maximum at about 660 nm. Using excitation at 530 nm and emission at 585 nm gave ethionine: control signals almost identical to what were obtained using the FLIPR.

Dihydrofluorescein Diacetate (DHFDA) Assay:

Autofluorescence of HepG2cells was determined using the FLIPR™. DHFDA (final 1 uM, 100 ul in Hanks Buffer, 2% bovine serum albumin, 0.01% Pluronic F127) was added to each well of treated HepG2 cells. After 1 hour, the plate was washed twice with 2% BSA in Hank's Buffer. Plate fluorescence readings were taken using the FLIPR™ over the next several days and compared to AlamarBlue™ fluorescent changes.

Assays with different drug exposure duration

Culture wells containing HepG2 cells were exposed to different drugs at various concentrations for either one day or for three days and then evaluated according to the traditional AlamarBlue assay. As seen in Figure 1A, AlamarBlue™ fluorescence responses from cells treated for 1 day (white bars) or 3 days (black bars) with cisplatin (cis-P), transplatin (trans-P), ethionine (ethio), methionine (methio), 5-fluorouracil (5FU), flufenamic acid (fluf), or diflunisal (diflu) are

generally more sensitive with the samples exposed to the toxins for three days. Control data are pooled from wells treated with media or 0.3% DMSO (vehicle for fluf and diflu). Figure 1B shows these same cell samples after a three day exposure to AlamarBlue™. Data are pooled from three experiments.

The traditional AlamarBlue™ assay (1 hour exposure to the dye, 1:100 dilution, 1%) gave strikingly different results for some toxic compounds depending on the duration of exposure (Fig. 1 A). When the duration of exposure was only 1 day, many compounds appeared less toxic (ie., the cells reduced more AlamarBlue™) than when examined after 3 days or longer (Fig. 1A). Prolonged exposure to AlamarBlue™ (using the same plates read for Fig. 1A) resulted in a very different set of results (Fig 1 B). In contrast to the decreased fluorescence observed with the traditional 1 hr exposure assay, cytotoxicity was indicated predominately by a pronounced INCREASE in AlamarBlue™ fluorescence after several days exposure to the dye (Fig. 1B). Toxicity was observed with short-term exposure to flufenamic acid and cisplatin, but prolonged exposure was required to see pronounced concentration-dependent effects of 5FU and ethionine (Fig. 1B). The fluorescence associated with non-toxic or untreated wells decreased by the second day of exposure to AlamarBlue™, while the increased fluorescence persisted in the toxic compound-treated wells; these differential changes greatly increased the signal associated with toxicity (Fig.2).

The increases in AlamarBlue™ fluorescence were reproducible both in time and magnitude for selected compounds. Highest fluorescence (>25,000 FLU) was observed with cytotoxins that acted rapidly: high concentrations (10 μg/ml) of cisplatin, the hepatotoxic non-steroidal antiinflammatory drugs, flufenamic acid and diflunisal, and the detergent Triton. However, even relatively small increases in fluorescence (>5,000 FLU, with 5FU for example) with prolonged AlamarBlue™

incubation were easily distinguished from control wells or relatively non-toxic analogues (typically 500-2,000 FLU).

Plates kept at room temperature showed essentially identical short-term AlamarBlue™ reduction. Long-term loss of control fluorescence occurred more slowly, but similar changes in fluorescence were observed at seven days at room temperature and three days at 37 C. Comparisons of AlamarBlue™ (1%) with resazurin (5 μM, a major component of AlamarBlue™), show little difference between the dyes in both traditional short-term exposure experiments (Fig. 3a) and in our novel long-term cytotoxicity assay (Fig. 3b). Resazurin reduction appears to account for the increased fluorescence observed with long-term AlamarBlue™ exposure.

EXAMPLE 2

Comparisons with the Oxidation-sensitive Dye Dihydrofluorescein Diacetate

Intracellular concentration and reduction of the colorless dye Dihydrofluorescein diacetate (DHFDA) to fluorescein by toxicant-treated HepG2 cells was compared to the long-term AlamarBlue™ reduction (Fig. 4A,B), with the idea that similar processes may be involved in fluorescence increases of both dyes. Addition of an antioxidant mixture (n-acetylcysteine 3 mM, tocopherol phosphate 100 uM, and ascorbate 100 uM) after 24 hours of toxicant exposure decreased basal DHFDA reduction in several experiments, but did not block the weak increased fluorescence observed with flufenamic acid, 5FU and diflunisal (Fig. 4A). Antioxidants did not consistently affect basal or toxicant-induced AlamarBlue™ reduction (Fig. 4B).

Effect of an Electron Coupler on the long-term Resazurin assay

PMS, an electron coupler that greatly increased the signal of other mitochondrial function (reductase) indicators such as XTT and WST-1, had a small

effect on the short-term AlamarBlue™ assay (Fig.5A). However, PMS dramatically increased the basal (but not toxicant-induced) fluorescence after long term AlamarBlue™ exposure, and thus eliminated the signal associated with toxicity (from over 20-fold to less than 2-fold, Fig. 5B). The increase in basal AlamarBlue™ reduction after addition of the electron coupler suggested that prolonged exposure to AlamarBlue™ or at least its reduced form might inhibit further production of reduced AlamarBlue™ in control cells. If this was the case, the effect appears to be largely reversible. Removal of AlamarBlue™ after seven days exposure, and replacement of fresh AlamarBlue™ and media (but with no replacement of toxic compounds) resulted in less sensitivity but similar short-term (Fig. 6A) and long-term fluorescent changes to those observed with AlamarBlue™-naïve cells (Fig. 6B).

EXAMPLE 3:

Comparisons of Toxicity in Fast and Slow-growing HepG2 Cells

Comparison of AlamarBlue™ reduction (increased fluorescence) in actively proliferating (50% confluent, white bars) and contact inhibited non-proliferating (100% confluent, black bars) HepG2 cells. Drugs were added for 1, 3 or 7 days prior to addition of AlamarBlue™. Fluorescence was determined after four days exposure to AlamarBlue™. Data were pooled from two experiments at each time point. Actively growing HepG2 cultures (treated at 50% confluence) were more affected by 5FU, ethionine and low concentrations of cisplatin than were slow growing cells (treated at 90% confluence, Fig. 7). These differences were most notable when AlamarBlue™ was added a day after treatment (Fig. 7A). More prolonged exposure to ethionine and 5FU produced much larger increases in AlamarBlue™ fluorescence, but the more confluent cells also showed increased responses (Fig. 7). When AlamarBlue™ was added a week after drug treatment, cytotoxic responses to low concentrations of drugs were increased and responses to high concentrations of drugs were diminished; thus the relationship between concentration and response

was not obvious (Fig. 7C). AlamarBlue™ reduction increased in the control confluent cell wells with prolonged drug incubation times and this increase may reflect death of cells (Fig. 7). Flufenamic acid and high concentrations of cisplatin produced pronounced responses regardless of growth stage, and the effects of the higher concentrations diminished over time regardless of confluence (Fig. 7). The control compounds transplatin and 5ClU produced minimal evidence of toxicity even when AlamarBlue™ was added after one week of treatment (Fig. 7C).

EXAMPLE 4

Long-term AlamarBlue™ Incubation with Primary Rat Hepatocytes

Similar to HepG2 cells, primary rat hepatocyte cultures reduced AlamarBlue™ to a more fluorescent form after exposure to toxic compounds. A time course for a typical response is shown in Fig. 8. There were similarities as well as differences in compound effects on primary rat hepatocytes and HepG2 cells. The non-steroidal antiinflammatory drugs diflunisal and flufenamic acid showed cytotoxicity at similar concentrations in both cultures. In contrast, aspirin appeared cytotoxic to rat hepatocytes, but not to HepG2 cells even at concentrations as high as 300 uM. Cisplatin was surprisingly potent in primary hepatocytes, showing effects at 3 uM, despite the non-proliferative state of these cells. Transplatin was minimally toxic at the same concentrations. Ethionine (3-30mM) was not toxic initially, but toxicity became apparent at later times as was observed in HepG2 cells; the control amino acid methionine (10 and 30mM) was not toxic.

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WHAT IS CLAIMED IS:

1. A method to determine the cytotoxicity of a test compound comprising the steps:

   (a) contacting cells in culture medium with a test compound;

   (b) culturing the cells in the presence of resazurin for a time sufficient for viable cells to metabolize resorfurin to reduce fluorescent emission at about 600nm;

   (c) measuring fluorescence of the cells; and

   (d) comparing the fluorescence of cells cultured without the test compound to the fluorescence of cells cultured with the test compound,

   wherein the cells have an increased fluorescence when cultured with a cytotoxic test compound compared to the fluorescence of cells cultured without a cytotoxic compound.

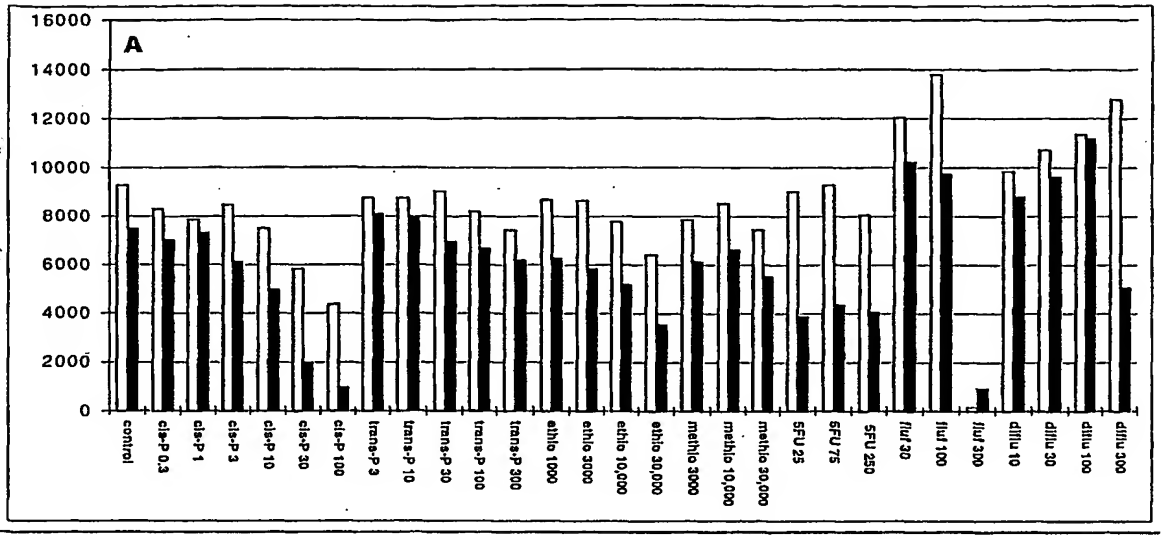
2. The method of claim 1 wherein the cells are selected from a group consisting of HepG2 cells primary human hepatocytes, and primary rat hepatocytes.

3. The method of claim 1 wherein the resazurin composition comprises resazurin as a single component in a physiological buffer.

4. The method of claim 1 wherein the resazurin composition is selected from a group consisting of AlamarBlue™, and a composition containing resazurin and poising agents.

5. The method of claim 1 wherein the time sufficient for the viable cells to oxidize the resorfurin is between about 20 to 72 hours.

FIGURE 1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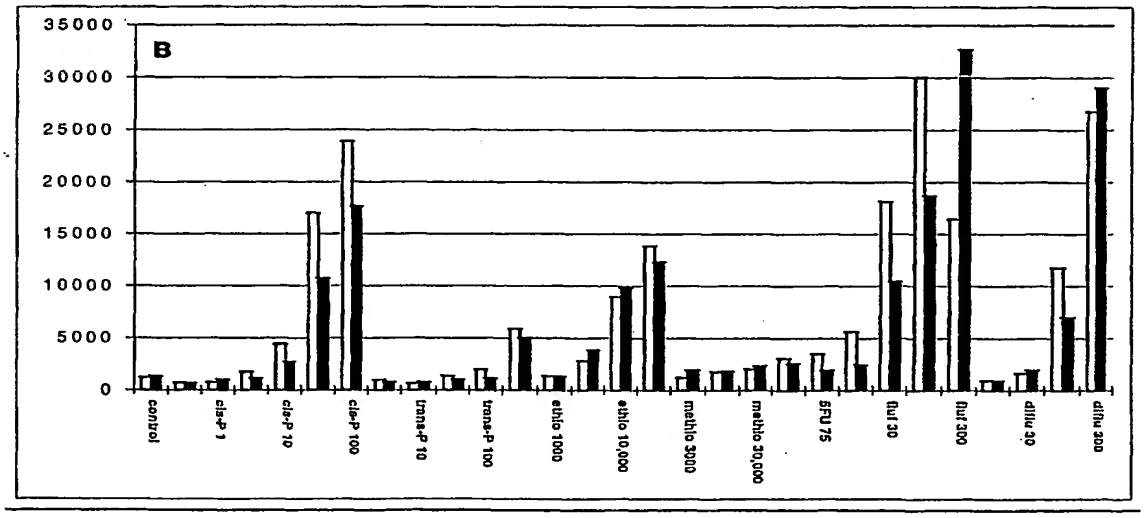
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FIGURE 2

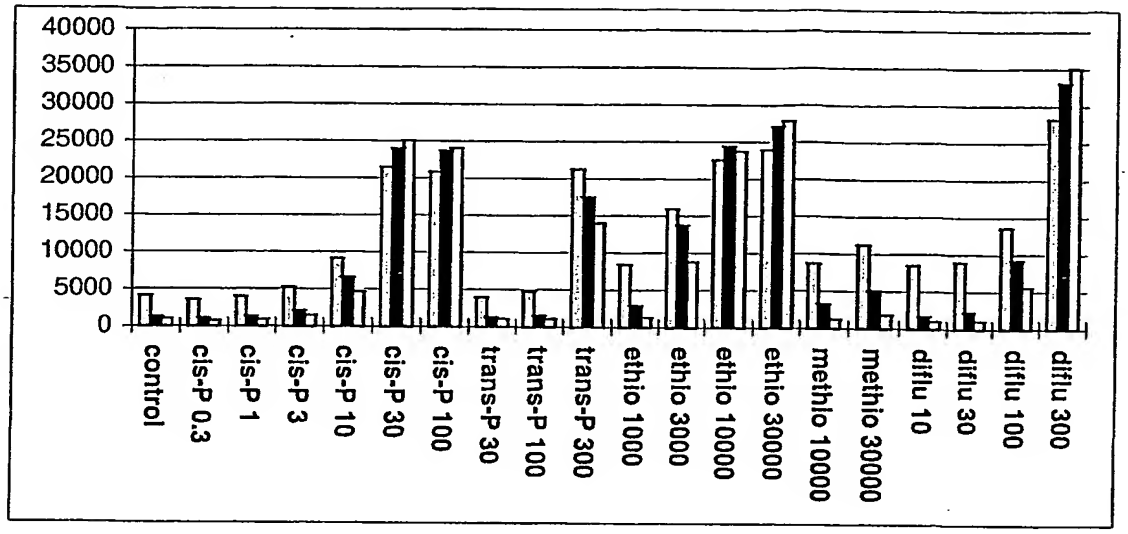
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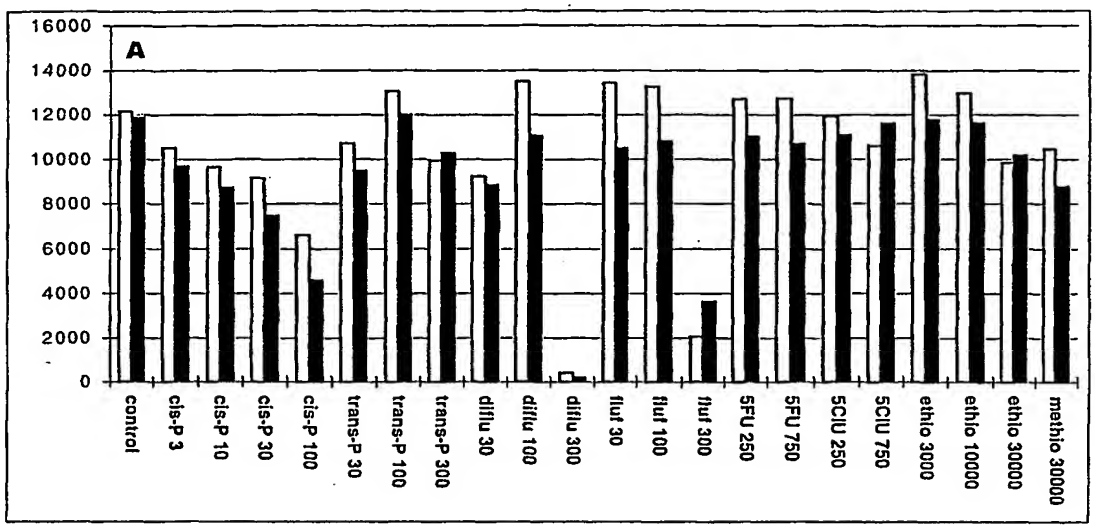
FIGURE 3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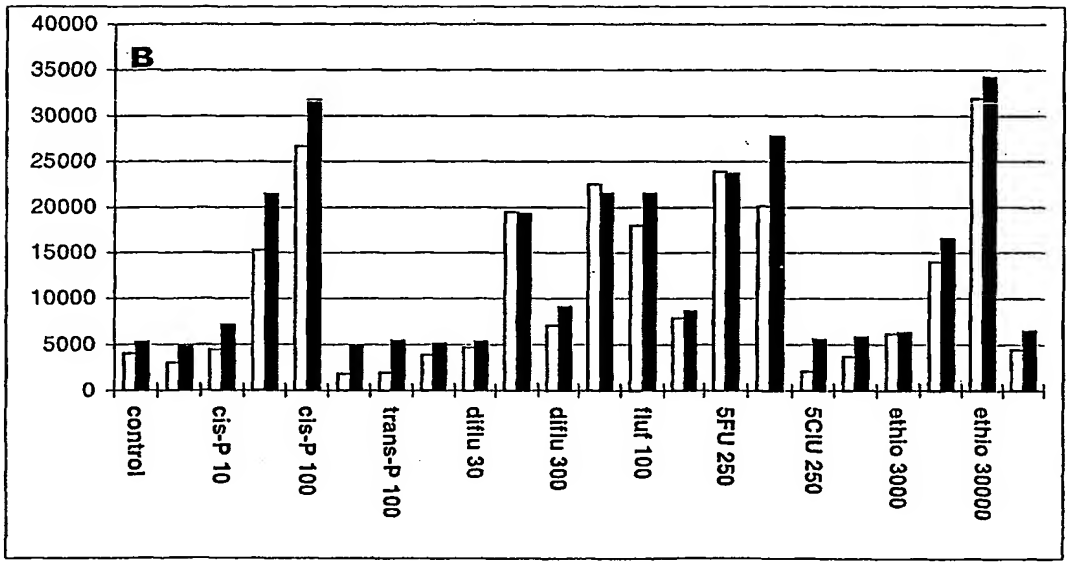
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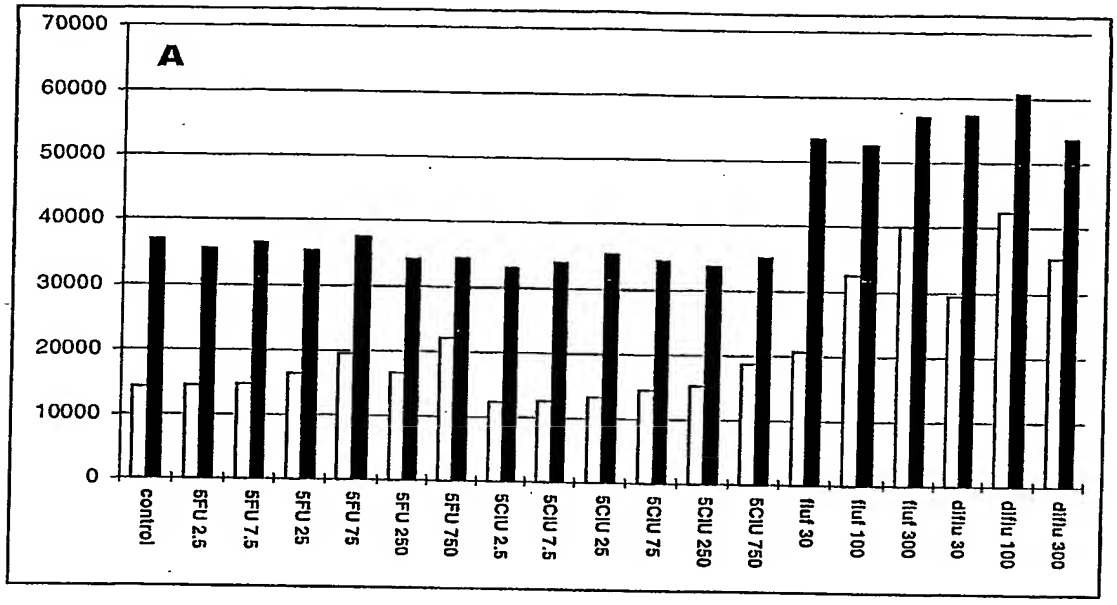
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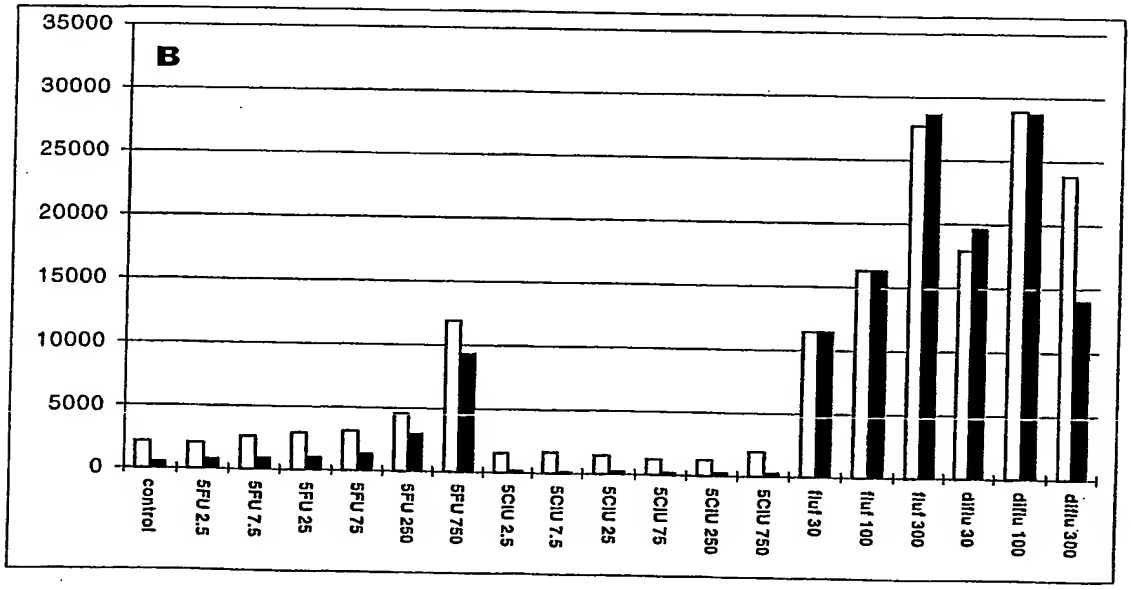
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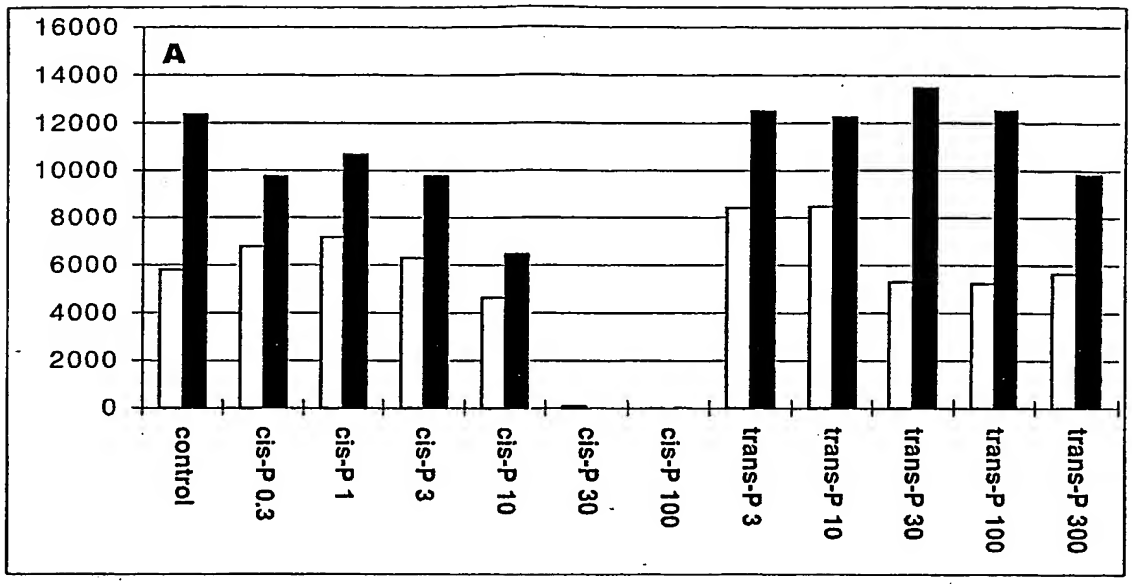
FIGURE 5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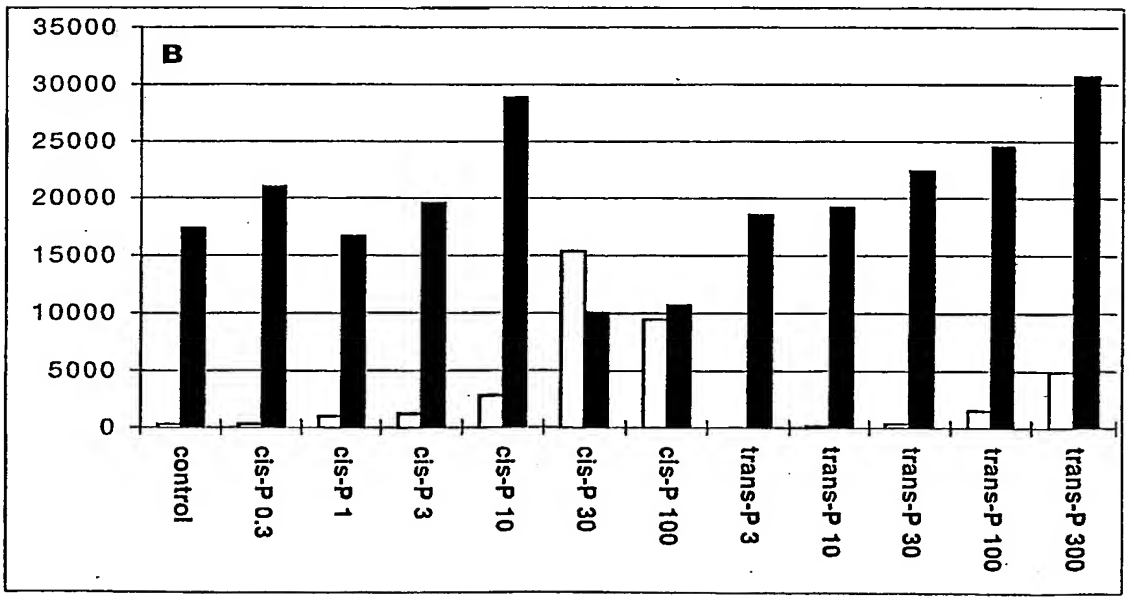
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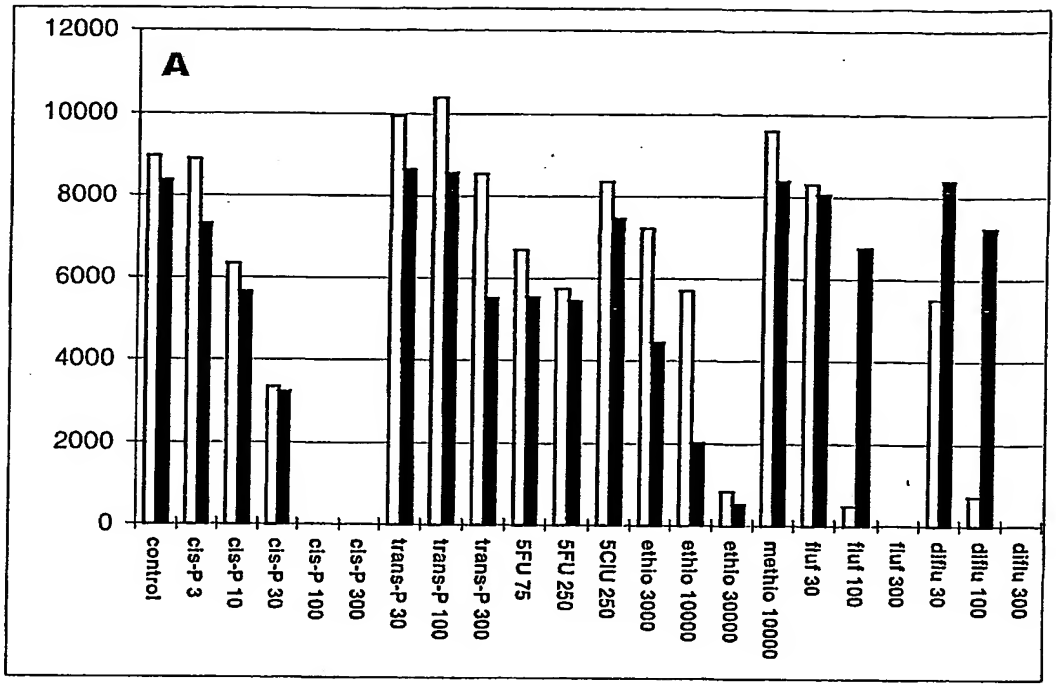
FIGURE 6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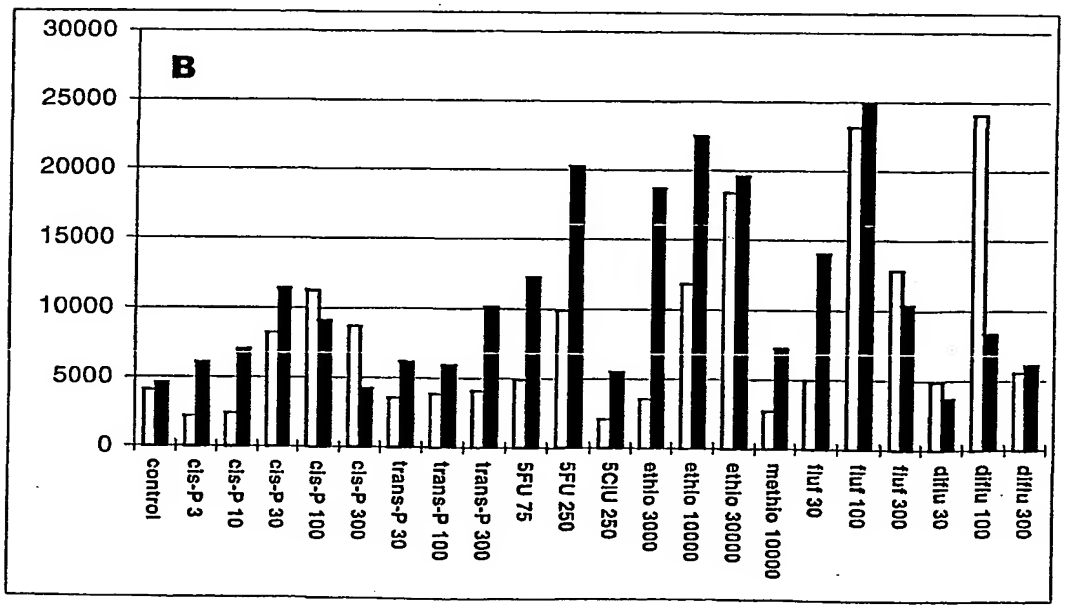
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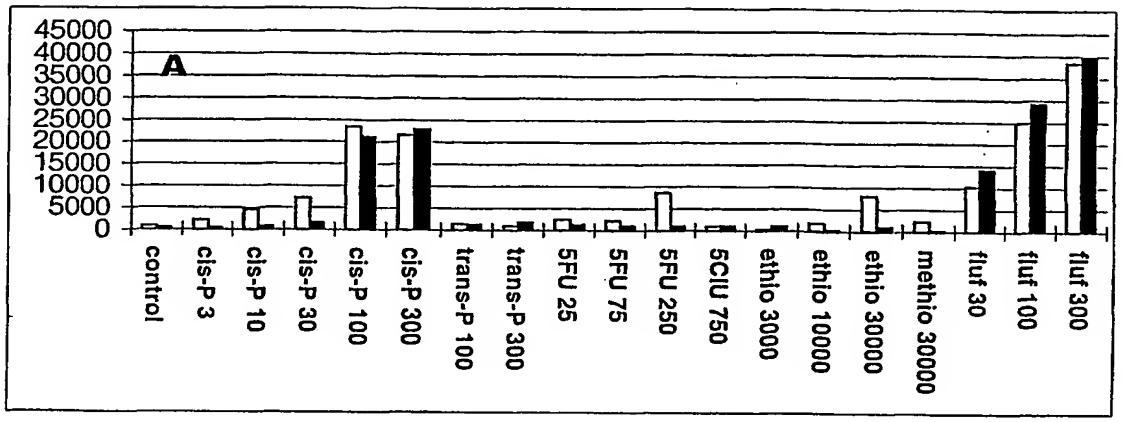
FIGURE 7
PANEL 1



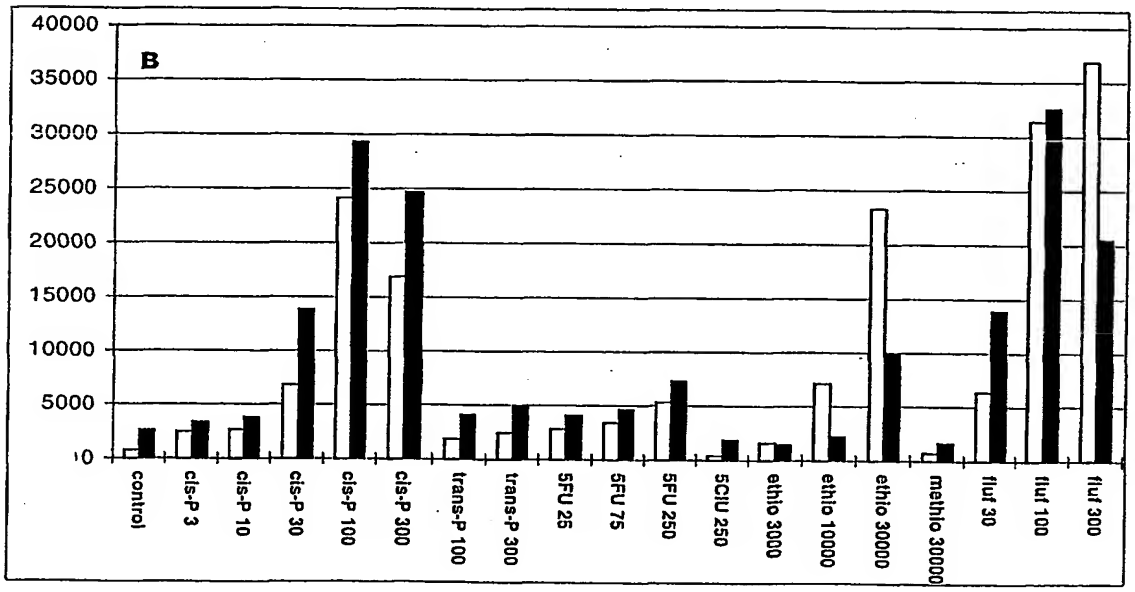


FIGURE 7
PANEL 2

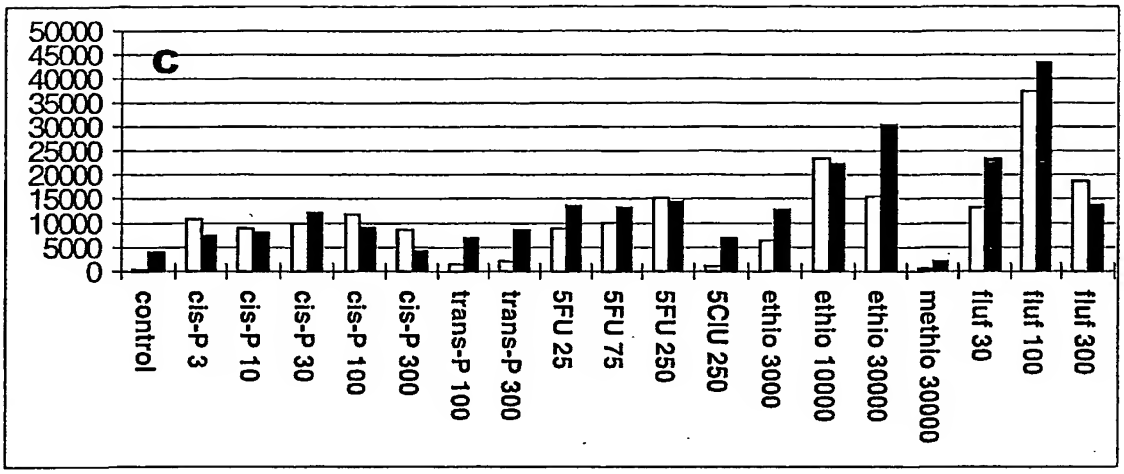
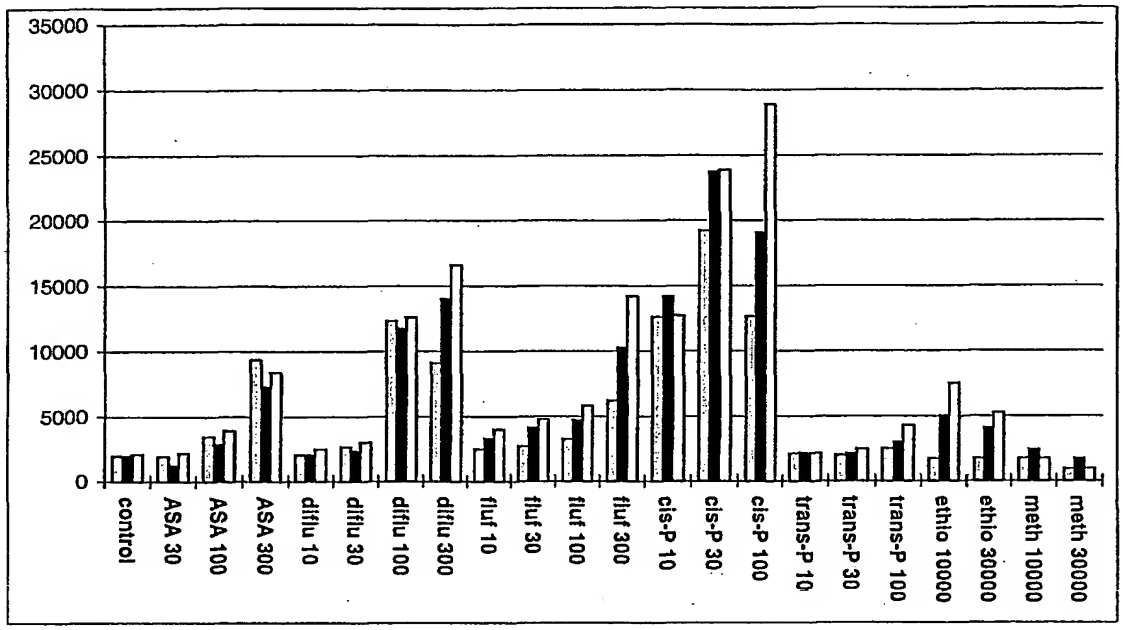


FIGURE 8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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/18570

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : C12Q 1/02, 1/08; C12N 1/00
US CL : 435/29, 32, 243
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 435/29, 32, 243

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MEDLINE, CAPLUS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	US 5,501,959 A (LANCASTER et al) 26 March 1996 (26.09.1996), column 3, lines 34-45; column 4, lines 26-45; column 23, lines 28-42, see entire document.	1, 4-5 --------- 2-3
X --- Y	US 5,858,974 A (LITTLE et al) 12 January 1999 (12.01.1999), column 23, lines 1-13, see entire document.	1, 3, 5 --------- 2, 4
X --- Y	US 5,756,527 A (MJALLI et al) 26 May 1998 (26. 05.1998), column 38, lines 54+, see entire document.	1, 3, 5 --------- 2, 4
X --- Y	AHMED, S.A. A new rapid and simple non-radioactive assay to monitor and determine the proliferation of lymphocytes: an alternative to [3H]thymidine incorporation assay. Journal of Immunologic Methods, 1994, Vol. 170, page 214, see entire document.	1, 3, 5 --------- 2, 4

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 August 2001 (01.08.2001)	07 AUG 2001
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703)305-3230	Authorized officer Mahreen Chaudhry Telephone No. (703) 308-1235

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US01/18570

C. (Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	PAGE, B et al. Sensitive colorimetric cytotoxicity measurement using Alamar Blue, Oncology Reports 1995, VOL. 2, pages 59-61, see entire document.	1, 3, 5 --- 2, 4
Y	Derwent Accession No. 1990-301035. JP 02211899A, 23 August 1990 (23.08.1990), see entire document.	1-5

Form PCT/ISA/210 (second sheet) (July 1998)